BACTERIAL DISSIMILATION OF CARBOHYDRATES[1]

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Received for publication October 7, 1939

Professor J. B. S. Haldane introduces his Sir F. Gowland Hopkins lecture (1937) on the Biochemistry of the Individual with the statement, "The ultimate aim of biochemistry may be stated as a complete account of intermediary metabolism, that is to say, of the transformations undergone by matter in passing through organisms." In this discussion, it is our purpose to reconstruct, in the light of present knowledge, the biochemical events occurring in the living bacterial cell concerned with the dissimilation of carbohydrates. Our knowledge is far from complete; however, "Although it is dangerous to speculate too far, it is foolish not to speculate at all" (Hill).

Our remarks will be more specifically directed toward the bacterial cell although the general problem of cell physiology has received more extensive treatment with yeast and with animal cells, especially those of muscle, brain and liver. There are certain advantages in dealing with metabolic phenomena in highly specialized cells of tissue such as muscle or brain rather than the bacterial cell. In the case of muscle or brain we are dealing with cells functioning in a well-protected and constant environment with respect to pH, temperature, redox conditions, nutritional properties and other factors; whereas, a bacterial cell is virtually a street urchin of the cell world; it is found functioning at temperatures between 80°C. and freezing; at pH levels of less than 1 to above 13; under strict anaerobiosis and strong aerobiosis; with a banquet before it at one time and again subsisting on a

[1] Journal Paper No. J-689 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 421.

diet relatively toxic. Accordingly, the bacterial cell is probably endowed with a large variety of enzymes and special mechanisms for use under widely differing conditions.

On the other hand, it is not improbable that bacteria offer marked advantages as material for study of the basic physiology of the living cell in its relationships to physical and chemical environment. More particularly those forms referred to as autotrophs show behaviors and characteristics, whose study will doubtless assist in providing an understanding of the primitive and unspecialized cell. According to generally accepted belief of biologists, life on earth is characterized by a gradual though distinct specialization of the simple forms of life to highly differentiated organisms. A spectrum may be visualized showing this transition from organisms requiring only an inorganic substrate for existence, on the one hand, to those extremely parasitic (differentiated) forms such as the viruses on the other. The degree of specialization may be indicated by the adaptability of the organism to its environment. The autotroph must have been among the early forms of life, a conception strengthened by the ease of adaptability to changing environment. In those prehistoric times bacteria must have been chemosynthetic, deriving their energy in the transfer of hydrogen to CO_2 (oligocarbophilous forms). It is likely that the use of oxygen is a more recent acquirement inasmuch as aerobes employ the anaerobic hydrogen-activating mechanism in connection with a coordinated aerobic mechanism which permits them to utilize O_2 as a hydrogen acceptor in place of some intermediately formed product as must an anaerobe. In this sense, an aerobe is an anaerobe which possesses an aerobic mechanism; therefore the anaerobic autotrophs must have preceded the aerobic autotrophs. Anaerobic dissimilation is simpler, less specialized and provides the energy requirements for organisms with low cellular differentiation. *Thiobacillus denitrificans* is an obligate anaerobic autotroph which oxidizes H_2S, S, thiosulfate or tetrathionate to sulfuric acid with the simultaneous reduction of CO_2 and NO_3. The methane bacteria are to be mentioned since some of these forms can obtain their energy chemosynthetically from the process:

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$. The better-known aerobic chemosynthetic autotrophs, owing to their utilization of O_2, obtain relatively more energy from their substrates. Illustrative of this group are *Thiobacillus thioparus* and *T. oxidans*.

One of the early distinct steps in differentiation occurred with the origin of the photosynthetic forms. When sunlight penetrated to the earth's surface, certain bacteria accepting the possibilities offered by radiant energy used chemical energy to synthesize what was perhaps the first photosynthetic bio-energy transformer. In this case, sunlight furnishes the energy to reduce CO_2 and during the process H_2S is oxidized to sulfur or its oxides. The existence of photosynthetic bacteria had been suspected since the work of Engelmann (1883, 1888) which described bacteria showing a well defined absorption spectrum. Every effort was made to show the liberation of gaseous oxygen; failing this and recognizing the necessity for H_2S, a chemosynthetic type of metabolism was postulated and gained acceptance in the belief that the dehydrogenation of H_2S provided the energy. Such an explanation, however, did not explain the rôle of light or account for the growth of the bacteria anaerobically. It remained for van Niel (1931, 1935) to reveal the true nature of the process and to harmonize the known facts. He showed that the purple sulfur bacteria oxidize H_2S stoichiometrically to sulfate: $H_2S + 2\ CO_2 + 2\ H_2O \rightarrow 2\ CH_2O + H_2SO_4$. In the case of the green bacteria the H_2S is oxidized only so far as sulfur: $2H_2S + CO_2 \rightarrow CH_2O + 2\ S + H_2O$. Roelofsen (1935) demonstrated an endogenous liberation of CO_2 in the dark; the CO_2 was immediately assimilated in the light in the presence of H_2S. van Niel has shown that no oxygen is liberated, and that indeed, no liberation of oxygen is to be expected but rather sulfur in the case of the green forms, or its oxides in the case of the purple sulfur bacteria. Members of both the purple sulfur bacteria (*Thiorhodaceae*) and purple bacteria (*Athiorhodaceae*) can utilize molecular hydrogen and certain organic donators.

Photosynthesis appears to show an intimate relationship of chemical and solar energy-yielding processes wherein chemical energy serves to reduce the CO_2 to carbohydrate and at the same

time oxidize the chlorophyll to dehydrochlorophyll (Conant, Dietz and Kamerling, 1931). Sunlight then furnishes the energy to regenerate the chlorophyll:

$$6CO_2 + 12 \text{ chlorophyll} \xrightarrow{\text{enzyme}} 12 \text{ dehydrochlorophyll} + C_6H_{12}O_6 + 6\ H_2O$$

$$12\ H_2O + 12 \text{ dehydrochlorophyll} \xrightarrow{\text{light}} 12 \text{ chlorophyll} + 6\ O_2$$

The use of sunlight was a great step in relieving the photosynthetic bacteria of chemosynthetic chores; the necessity of providing energy was thus solved by organisms "clever" enough to use chemical energy to synthesize a converter capable of utilizing solar radiation. Since van Niel (1931) has shown that green and purple sulfur bacteria are able to reduce CO_2 by means of hydrogen from H_2S with the assistance of radiant energy, the development of unicellular chlorophyll-containing organisms would be but a step in phylogenetic development.

The gradation of bacterial metabolism may be further extended. We find the facultative heterotrophic forms which function as autotrophs but can use more complex compounds as sources of either nitrogen or carbon or both, and the facultative autotrophs preferring complex sources of nitrogen and carbon but still able to use inorganic sources. Perhaps these two groups should be recognized as one, i.e., the facultative forms, but the important point is that as the spectrum is extended from the obligate autotrophs to the obligate heterotrophs, the specialization is marked by a gradual and progressive loss of certain synthetic properties. The utilization of CO_2 by the propionic acid bacteria (Wood and Werkman, 1936, 1938) may represent a vestige of autotrophism in otherwise heterotrophic organisms.

Synthesis of chlorophyll may have been an extremely important event since it would have made free oxygen available as a hydrogen acceptor. That is, it became possible for aerobic forms of life to develop; and the use of free oxygen greatly increased the energy economy of the cell. It is likely that differentiation of the anaerobe into an oxygen-utilizing type led to the

development of series of reversible graded energy systems which released energy for use of the organism in convenient quantities resulting in a smooth, even flow. With increasing differentiation the number of oxidation-reduction systems employed by a cell became greater and successive systems involved more components to yield a smoother flow of energy.

However, differentiation and specialization among the aerobic forms also must have occurred in the course of phylogenetic development. Among the first aerobes were those which used oxygen directly as a hydrogen acceptor. This simplest of aerobic oxidation mechanisms would result in a sudden and uneconomic release of energy. It would be interesting to search for simple oxytropic systems in the primitive bacteria. If such systems do still exist, they must be rapidly disappearing with development of specialization and differentiation. Later, there developed more efficient yet more specialized types of respiration employing hydrogen carriers such as respiratory pigments and flavoproteins, and still later, types such as the cytochrome-containing forms requiring two or more carriers and oxidases.

Certain bacteria require small amounts of a considerable number of growth substances (dissimilation and assimilation factors) in their metabolism exclusive of proteins and carbohydrates. Illustrative of this group are: thiamin (as cocarboxylase) essential in pyruvate metabolism, riboflavin (yellow enzyme group), and nicotinic acid or its amide in hydrogen transportation, adenylic acid and its diphosphate and triphosphate in phosphorylation, vitamin B_6 and pantothenic acid whose functions are unknown at present.

Obviously since the autotrophic organisms do not require the addition of such factors to the medium, they must either synthesize their own or do not require them in their metabolism. Evidence supports the former assumption although instances probably may be found in which the latter assumption applies. *Aerobacter aerogenes* and *Escherichia coli* synthesize thiamin so rapidly that they cannot be depleted by growing on a thiamin-free medium, whereas the more fastidious *Propionibacterium pentosaceum* shows great stimulation on addition of thiamin to cultures

which have been grown on a medium rich in the vitamin and then transferred to a vitamin-deficient medium for not to exceed three successive transfers (Silverman and Werkman, 1938). After additional transfers *Propionibacterium pentosaceum* acquires the ability to synthesize its thiamin. The more parasitic organisms require the addition of the various factors to a synthetic medium, whereas probably many of the factors necessary for growth of the extreme parasites in artificial medium are as yet unknown. The "growth factor" requirements of organisms of this portion of the spectrum probably will be greatly extended.

Bacteria show marked ability to adjust themselves to their environment by selection and by mutation. Dissimilation of specific carbohydrates can no longer be as rigorously accepted in differentiating species or genera as in the past. Bacterial variability reminiscent of a primitive nature is of the greatest value in the study of cell genetics.

Stephenson and her coworkers have demonstrated that adaptation can occur in the parent cell, and it is probably not necessary that the cell multiply in the presence of the specific substrate in order to produce the enzyme for its breakdown. In this case, the addition of the specific substrate to non-proliferating cells is sufficient to evoke the enzyme. In other cases, the presence of the specific substrate has little to do with the appearance of the enzyme which may be effected by other factors. Thus alanine deaminase in *Escherichia coli* is not increased by the presence of alanine and may be all but eliminated by the presence of glucose (Stephenson and Gale, 1937).

It is our experience (Wiggert and Werkman, 1939) that two distinct physiological types of cells of *Propionibacterium pentosaceum* differing in sodium fluoride sensitivity and, more important, in ability to ferment phosphoglyceric acid, result from culturing in the presence and in the absence of sodium fluoride. Bacterial variation must of course be differentiated from effects due to variation in physical and chemical environment during culturing. Since mere traces of elements or compounds may be all that is required for a luxuriant growth or activity, an apparent

variation in a microorganism may result from unintentional differences in the medium.

DISSIMILATION OF CARBOHYDRATES

For present purposes, bacterial metabolism will be discussed as schematized in figure 1. The discussion will emphasize dissimilation, which may be defined as the transformation of the substrate to yield energy to the organism, as distinguished from those endothermic changes which characterize assimilation. Dis-

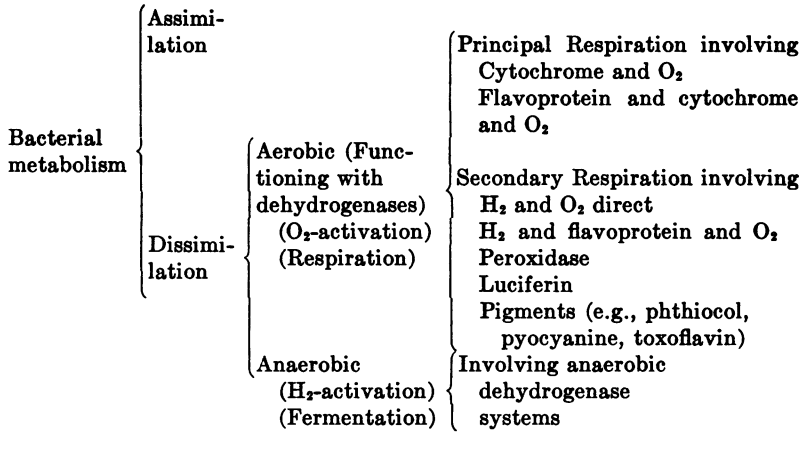


FIG. 1. SCHEMA OF BACTERIAL METABOLISM

similation may, of course, also furnish certain intermediate products necessary as building blocks in assimilation.

For convenience of discussion, we shall speak of aerobic dissimilation and anaerobic dissimilation. The former is respiration, the latter is fermentation in the Pasteurian sense. Both are truly oxidative processes.

We owe much to the genius of Pasteur who recognized that the absence of a respiratory process resulted in "la fermentation, c'est la vie sans air." It was Pasteur who recognized the *physiological equivalence* of fermentation and respiration.

Pasteur (1876) in giving a broad interpretation of what is now known as the Pasteur effect stated (free translation): "Fermenta-

tion is a very general phenomenon. It is life without air, life without free oxygen, or in more general terms, it is the result of a chemical process on a fermentable substance, i.e., capable of producing heat by decomposition."

"Fermentation—a chemical process, connected with the vegetative life of cells—takes place at a moment when these cells, ceasing to have the ability of freely consuming their substrate by respiratory processes—that is, by the absorption of free oxygen —continue to live by utilizing oxygenated substances like sugar. This characteristic (of fermentation) is always ready to manifest itself and in reality does so as soon as life ceases to perform its functions under the influence of free oxygen or without a quantity of that gas sufficient for all acts of nutrition."

Pasteur, with keen insight, has thus given us a concise picture and it is convenient to recognize his differentiation. His general concept has required modification only in one respect in which subsequent investigation has shown lack of breadth, i.e., his insistent demand that the phenomena of fermentation are correlative with life or vital activity (cf. Burk, 1937). Buchner's preparation of an active "press-juice" in 1897 did much to push Pasteur's views into the background for two decades. However, in this respect it should be noted that to date no active preparation, free of cells or cell fragments, of the respiratory enzymes has as yet been obtained. At any rate, the wisdom of Pasteur did not prevent an undue emphasis being placed on the rôle of oxygen in biological oxidation after the discovery of oxygen by Priestley and the enunciations of Lavoisier.

The terms respiration and fermentation have been variously defined and used, even by the same author. It is not so important whether one or another term is used, as it is that the term employed be adequately defined in the light of our present knowledge. Differentiation of the two processes is solely a matter of convenience and no fundamental difference is implied. The intimate relationship of fermentation and respiration is revealed in the Pasteur reaction, a phenomenon which has not been adequately elucidated but does show the quantitative inter-

dependence of the two processes. The linkage between the two reactions has never been discovered although it is generally accepted that aerobic dissimilation is always associated with fermentation, i.e., aerobic carbohydrate dissimilation is always preceded by anaerobic phases (phosphorylation and splitting), in spite of the discovery of enzymes oxidizing glucose directly, or that monoiodoacetic acid at certain concentrations inhibits fermentation and not respiration.

The allocations under Principal and Secondary respiration (fig. 1) are largely speculative and are based on results obtained with cells other than bacteria. They are given here for completeness. Future investigation will show the proper allocations and provide details of mechanism.

Biological oxidation

It is now generally accepted that biological oxidation-reduction manifests itself as a transfer of hydrogen (electrons) from donator to an acceptor, the transfer yielding energy to the organism. Clark (1923) has adequately defined biological oxidation as "the withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen." The rôle of oxygen is that of a hydrogen acceptor. Under anaerobic conditions the O_2 is replaced by some other suitable H_2-acceptor. Thunberg (1917, 1918, 1920) and Wieland (1912, 1913, 1922a, 1922b, 1925) have provided us with the basic concepts of our present knowledge of biological oxidation-reduction.

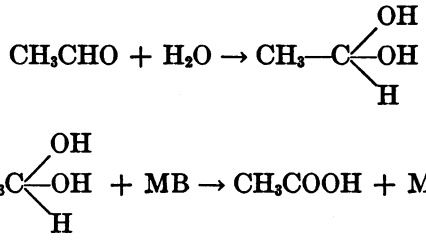
The transfer of hydrogen is activated by specific enzymes known as dehydrogenases. Wieland's original concept did not provide for the activation of the H_2-acceptor but only of the donator. It is apparent that certain of the acceptors do not require activation; e.g., methylene blue. On the other hand, probably most naturally occurring acceptors do require activation. The term "activation" has not been defined; nor will any rigid definition be attempted. For the time being, we must sense the mean-

ing. Suffice it to illustrate the point: If succinic acid and methylene blue coexist in solution, no detectable chemical change occurs even after long time, i.e., the substrate is stable even in the presence of oxygen; however, on addition of an active suspension of washed cells of *Escherichia coli*, the methylene blue accepts hydrogen to become reduced methylene blue and succinic acid is dehydrogenated to form fumaric acid. In this case the succinic acid molecule has been activated to donate hydrogen to methylene blue. The trigger-like activation has initiated the the transfer of hydrogen. The term activation is used to express a general concept, i.e., that certain metabolites such as glucose, succinic acid, lactic acid, polyalcohols, etc., are so changed under the influence of certain specific cellular agents that hydrogen atoms may become transferred to reducible substances (H_2-acceptors), a transfer which otherwise would not occur (Thunberg, 1937). The phenomenon requires a designation, and the term activation is as convenient as any and does not imply the nature of the mechanism responsible (cf. Michaelis, 1933). Not only is activation necessary but the donator and acceptor must be suitable, i.e., the transfer of hydrogen must result in a decrease in the free energy of the system. It should be emphasized that the transfer of hydrogen anaerobically is as truly an oxidation as its transfer aerobically where O_2 is the acceptor. It is now becoming clear that this transfer occurs in orderly fashion and has a complex mechanism. It may occur between molecules or within the same molecule and may be symbolized thus:

$$AH_2 + B \xrightarrow{\text{dehydrogenase}} A + BH_2 \text{ (or } ABH_2\text{)}$$

$$ABH_2 \xrightarrow{\text{dehydrogenase}} AH_2B \text{(or } AH_2 + B\text{)}$$

Wieland (1925) provided support for his theory by demonstrating the oxidation of acetaldehyde to acetic acid by *Acetobacter* in the absence of free oxygen with methylene blue as the hydrogen acceptor. In this case, the aldehyde is hydrated and two atoms of hydrogen are activated by a dehydrogenase and transferred to the methylene blue.



Dehydrogenases. Ehrlich as early as 1885 injected methylene blue into animal tissues and found that most of them reduced the dye. Schardinger in 1902 discovered an enzyme in milk which reduced methylene blue in the presence of an aldehyde. It was not until a decade later that the significance of the action of such enzymes was realized and dehydrogenases were recognized as enzymes activating the release of hydrogen from the molecule of the donator, the hydrogen being transferred to an acceptor. Thunberg's methylene blue technique (cf. Ahlgren, 1936) is generally used to demonstrate the presence of a dehydrogenase. Dehydrogenases may function in the transfer of hydrogen from the donator directly to oxygen as the acceptor. Such dehydrogenases are called oxytropic dehydrogenases and apparently form H_2O_2. It is difficult to differentiate the oxytropic dehydrogenases and the oxidases. Perhaps no harm will result if we think of the dehydrogenases as activating the release of hydrogen, the oxidases as activating the oxygen as an acceptor.

Oxytropic dehydrogenases function with suitable dyes as acceptors in the absence of O_2 and may, or may not, be cyanide-sensitive. They do not require coenzymes or the cytochrome-oxidase system, and may be considered relatively simple systems in that the activated hydrogen passes directly from the donator to O_2 without recourse to carrier systems.

The anaerobic dehydrogenases are those capable of activating the release of hydrogen in a system in which the immediate acceptor is not molecular oxygen. Certain of the anaerobic dehydrogenases function through coenzymes which may in turn require a dehydrogenase, e.g., diaphorase, to activate the release of hydrogen from the reduced coenzyme. The route of hydrogen

transfer is generally devious and may not be a single or fixed path; it is not unlikely that any naturally occurring reversibly oxidizable and reducible substance of suitable potential may function as a transporter of hydrogen. Among those occurring in bacteria are riboflavin, cytochrome, coenzyme I and II, pyocyanine, phthiocol and toxoflavin. For a summary of dehydrogenase systems and bacterial fermentations the reviews of Kluyver (1935), Harrison (1935) and Potter (1939) may be consulted.

Coenzymes. In addition to the hydrogen donator, the hydrogen acceptor, necessary dehydrogenases and supplementary factors such as water, buffers and inorganic ions, many biological reactions require the presence of coenzymes. A coenzyme may be defined as a dialyzable, thermostable substance necessary in addition to the enzyme and substrate to initiate a reaction. A coenzyme is usually an organic compound although the term was first used by Bertrand (1897) to characterize inorganic ions (Ca and Mn) which activated plant enzymes. The present usage dates from the work of Harden and Young (1905, 1906) who found the thermostable, dialyzable fraction of yeast-juice necessary to initiate fermentation in the residue. Considerable confusion existed regarding the nature of yeast coenzyme (cozymase). In fact any dialyzable, thermostable substance stimulating the action of yeast press-juice was considered a coenzyme; i.e., Mg, K, PO_4, hydrogen acceptor (necessary to initiate certain reactions which continue by virtue of acceptors formed subsequently), anti-protease and Euler's principle. Owing to the work of Euler (1936) and Warburg in recent years, remarkable progress has been made in elucidating the function of coenzymes. Cozymase I of Euler and cozymase II of Warburg and Christian, both of which are adenylic acid nucleotides of nicotinic acid amide, are important H_2-carriers, capable of passing hydrogen to flavoprotein. Coenzyme II acts with hexosemonophosphate dehydrogenase; coenzyme I acts with hexosediphosphate, lactic acid (muscle), alcohol and malic acid dehydrogenases. Recently Euler and Adler (1938) have shown the biological inter-conversion of the two coenzymes.

A coenzyme may function in one or more of several ways; i.e., as a H_2-carrier, phosphate-carrier, oxygen-carrier or in ways not now clear. Euler and Myrbäck (1923) proposed the term cozymase for the coenzyme of alcoholic fermentation, whereas the enzyme free from coenzyme has been termed apozymase (Neuberg and Euler, 1931). The present tendency is to use the term coenzyme in referring to thermostable, dialyzable organic substances; e.g., Harden and Young's coenzyme (cozymase, codehydrase, coenzyme I, diphosphopyridine nucleotide), coenzyme II (triphosphopyridine nucleotide), cocarboxylase (thiamin pyrophosphate), and adenylic acid.

Although coenzymes were formerly looked upon as separate entities which accelerated the reactions brought about by the enzymes proper, our point of view is changing. The relationship may involve a union of coenzyme and apoenzyme to form the enzyme (holoenzyme); the coenzyme being a prosthetic group. On the other hand, the coenzyme may constitute a relatively separate entity. In view of recent results, both concepts or a modification may apply. It is probable that in certain cases the coenzyme is very easily dissociated, whereas with other enzymes dissociation does not occur with our present methods. The latter would be represented by those enzymes now considered as not requiring coenzymes. Theorell (1935a) concluded that the dehydrogenase combined with the substrate but not with coenzyme (coenzyme II) although the latter did combine with the substrate-enzyme complex. On the contrary Warburg (1928) insists upon the union of enzyme and coenzyme. Theorell (1935b) split the riboflavin-protein into a protein and a prosthetic group by dialysis. The protein could be combined with flavin phosphate prepared synthetically to form an active enzyme. In a number of cases the protein has been combined with a prosthetic group to yield an active enzyme. It seems that there is ample evidence that it is a question of ease of separation of the holoenzyme (proteid of Warburg) into coenzyme and apoenzyme. As new methods of separation are developed it will be found probably, that enzymes now considered to require no coenzyme will be dissociated into coenzyme and protein carrier.

There is evidence that the pyridine nucleotides (coenzymes I and II) also combine with specific proteins to form active complexes but that the latter are easily dissociated, more so than the flavin proteins.

A cell provided with suitable dehydrogenases with necessary coenzymes, H_2-donators and H_2-acceptors is still not equipped for dissimilation. The evidence seems clear that phosphorylation plays an essential rôle in the dissimilation of glucose by the normal cell and that phosphate-carrying coenzymes are necessary. Although it was formerly believed that the rôle of phosphoryla-

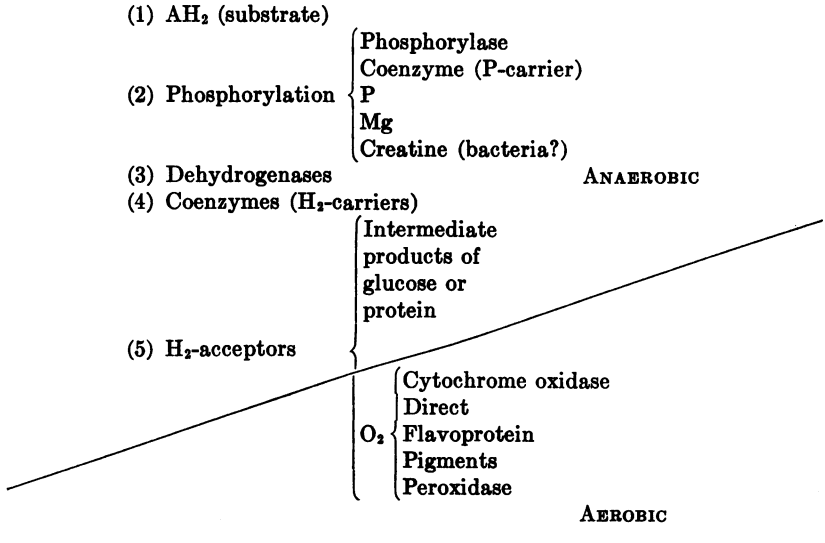


FIG. 2. COMPONENTS OF AEROBIC AND ANAEROBIC DISSIMILATION

tion was limited to the early stages of glycolysis, it is now certain that it is essential in all but the last steps following pyruvic acid. It is possible that bacteria and fungi possess in addition, a mechanism to utilize carbohydrates not involving phosphorylation (cf. Nord, Dammann and Hofstetter, 1936). Wiggert and Werkman (1938) have recently shown the association of phosphorylation of glucose and glucolysis in the living bacterial cell. Phosphorylation of glucose by tissue and yeast cells is generally accepted (Macfarlane, 1936).

A cell may be able to live an anaerobic existence from the point of view discussed; however, it may have learned also to use molecular oxygen as a hydrogen acceptor.

In figure 2 are illustrated the various types of factors necessary in anaerobic and aerobic dissimilation. The fact must be kept in mind that aerobic dissimilation is conditioned on the presence of suitable dehydrogenase systems.

Aerobic dissimilation is more economical than anaerobic dissimilation in the sense that the molal energy of glucose dissimilation by O_2 to CO_2 and H_2O is approximately a dozen times greater than the anaerobic dissimilation leading only to lactic acid as the final product.

The lactic acid fermentation may serve as an example of anaerobic behavior. We observe that much of the energy represented by the sugar remains locked within the two molecules of lactic acid (cf. Burk, 1937). This process is uneconomical. Now let us assume that the dissimilation occurs in the presence of oxygen as a hydrogen acceptor, and that it results in the formation of CO_2 and H_2O.

Anaerobic Dissimilation Involving Lactic Acid

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$$
$$-\Delta F = 54{,}740 \text{ calories}$$

Aerobic Dissimilation to Carbon Dioxide and Water

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CHOHCOOH$$
$$2\ CH_3CHOHCOOH + \tfrac{1}{2}\ O_2 \rightarrow CH_3COCOOH + H_2O$$
$$CH_3COCOOH + \tfrac{1}{2}\ O_2 \rightarrow CH_3COOH + CO_2$$
$$CH_3COOH + 2\ O_2 \rightarrow 2\ CO_2 + 2\ H_2O$$
$$-\Delta F = 664{,}000 \text{ calories}$$

It should be recalled that the energy released by dissimilation remains constant, so long as the final products remain the same, i.e., the available energy is independent of the path of the intermediary breakdown.

Mechanism of anaerobic dissimilation (fermentation)

It is convenient to discuss first anaerobic dissimilation and secondly aerobic dissimilation inasmuch as the aerobes appear to have developed from the anaerobes.

Bacterial dissimilation generally involves transformations in the glucose molecule or a polymer as a source of energy. For this reason glucose will serve as the substrate in the discussion of anaerobic dissimilation, and certain analogies will be drawn with schemes of metabolism which have been proposed for muscle and yeast in particular. Recent progress in our knowledge of the biochemistry of muscle metabolism has led to marked changes in our views regarding the dissimilation of carbohydrates by microorganisms, especially the heterotrophic forms.

Our knowledge of the intermediary metabolism of the autotrophic bacteria is quite fragmentary. The metabolism of the heterotrophic and more highly specialized forms bears at least in part, a similarity to that of animal cells with regard to enzymes, coenzymes, carriers and other agents. We have little information regarding the autotrophic intermediary mechanism. Our discussion then is concerned more specifically with bacteria belonging to the heterotrophic *Eubacteriales.*

Numerous theories have been proposed to account for cellular dissimilation; it has been well established that the living cell must have energy to carry on its metabolism, grow and reproduce; this energy is furnished stored in the food molecule which must be rearranged to provide available energy to the cell. This is, of course, excluding the photosynthetic bacteria which utilize the energy of the sun, as do typical chlorophyll-containing plants.

The Embden-Meyerhof-Parnas theory. The work of Embden, Deuticke and Kraft (1933) dealt with muscle metabolism. Meyerhof greatly expanded the work with muscle and extended Embden's theory to yeast. The investigations of these workers in the field of muscle and yeast metabolism have proved of inestimable value in the field of bacterial metabolism. Werkman and coworkers have presented experimental evidence that the Embden-Meyerhof-Parnas theory finds application to bacteria by their isolation of the key intermediate of that scheme (phosphoglyceric acid) from a wide variety of bacterial fermentations. Phosphoglyceric acid was first isolated in the case of bacteria from *Citrobacter freundii* (Werkman, Zoellner, Gilman and Reynolds, 1936) and later from *Escherichia, Aerobacter, Propioni-*

bacterium (Stone and Werkman, 1936a, 1936b), and organisms of a relatively large number of genera: *Bacillus, Azotobacter, Serratia, Lactobacillus, Streptococcus* and *Staphylococcus* (Stone and Werkman, 1937; Werkman, Stone and Wood, 1937). Recently Endo (1938) in Meyerhof's laboratory has confirmed and extended the work in the case of *E. coli.*

Much of the work has been carried out on cell-free juices of yeast and muscle. Satisfactory bacterial juices are difficult to prepare. Booth and Green (1938) have prepared an active juice in a roller grinder and Wiggert, Silverman, Utter and Werkman (1939) have obtained an active preparation by grinding the cells with powdered glass and centrifuging the resulting juice in a Beams ultracentrifuge. Such an active juice can be dialyzed and essential coenzymes thus removed, studied and identified. In addition to dialysis, specific inhibitors can be used to suppress certain reactions leaving others active; also, certain agents may be used to fix intermediate products for identification. Finally one arrives at results from which a general scheme of fermentation can be synthesized. Silverman and Werkman (unpublished) have prepared a cell-free juice from *Aerobacter aerogenes* capable of making the following conversion: $2\ CH_3COCOOH \rightarrow 2\ CO_2 + CH_3COCHOHCH_3$. The simple system may provide a method of attack leading to the elucidation of the synthesis of 4-carbon compounds from 3-carbon substrates. The use of dialyzed cell-free juices should permit us to reconstruct the biochemical events occurring in bacterial dissimilation. This method of attack has proved profitable in studies on animal and yeast metabolism; it should prove equally valuable in studies on bacteria (cf. Parnas, 1938a).

The scheme of Embden, Meyerhof and Parnas is given in figure 3 for reference. The reactions consist of oxidations, hydrolyses and phosphorylations.

It is generally accepted that cellular utilization of glucose involves its phosphorylation with a subsequent split into phosphorylated trioses. Hevesy (1938) and Parnas (1938b) have conducted experiments with tissues, eggs, milk and yeast fermentations with synthetic radioactive adenylic acid, in which it

was possible to trace the radioactive phosphorus. After a period of incubation, a considerable portion of the active phosphorus was found in the sugar-phosphoric acid ester fraction. Evidence indicates that this general scheme of events occurs with the bacterial cell as well as with muscle and yeast. The evidence is clear that phosphorylated hexoses occur in bacterial metabolism (Virtanen, 1924, 1925; Virtanen and Karström, 1931). Adenylic

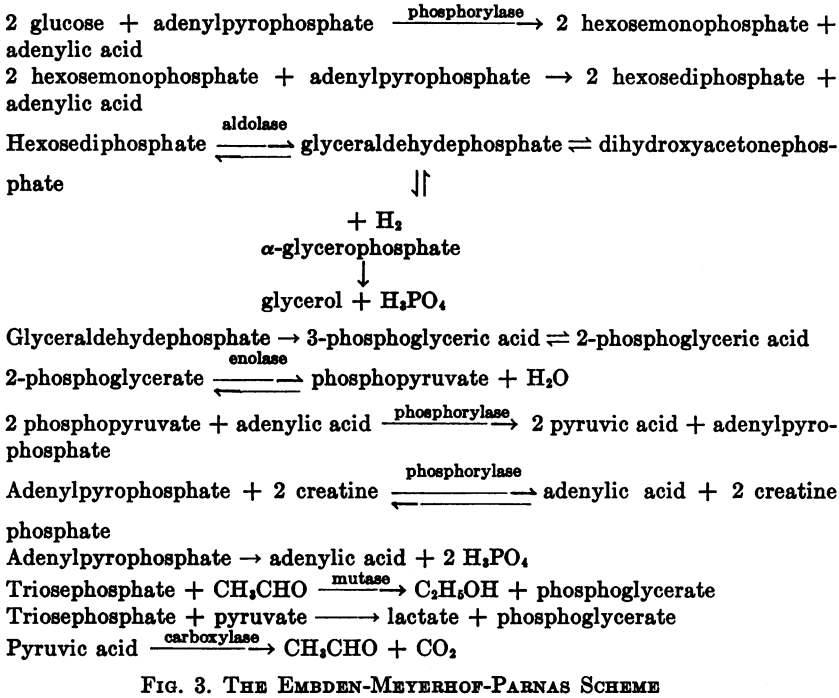


FIG. 3. THE EMBDEN-MEYERHOF-PARNAS SCHEME

acid and its allied phosphates act as coenzymes in the transfer of phosphate from phosphopyruvic acid to glucose. Among these adenosine nucleotides are: adenylic acid, adenosine diphosphoric and triphosphoric acids, diadenosine pentaphosphoric acid, diadenosine tetraphosphoric acid and the pyrophosphate (Kiessling and Meyerhof, 1938); all apparently function as phosphate carriers in cellular glycolysis although the active participation of all in bacterial metabolism has not been established.

Phosphorylation of glucose leads to the formation of hexose-6-phosphate which is an equilibrium mixture: glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate. There is as yet no evidence for the formation of the Cori ester (hexose-1-phosphate) (Cori and Cori, 1936, 1937) by bacteria. This ester is formed from the polysaccharides and rapidly converted into the 6-ester. The 6-monophosphate is converted into the Harden-Young ester (fructose-1, 6-diphosphate). Phosphorylation of the hexose leads by an intramolecular rearrangement to an "active" form of the sugar which is indicated to have a furanoid structure by the fact that the cardinal intermediate hexose (fructose-1, 6-diphosphate) is a furanoid. The diphosphate is the precursor of all triosephosphate which reacts with cozymase (coenzyme I). This reduction proceeds slowly (Meyerhof, 1938) unless a phosphate acceptor is present.

In most tissues the hexosediphosphate is thus converted into a complex equilibrium mixture:

$$\text{hexosediphosphate} \xrightleftharpoons{\text{aldolase}} \text{glyceric aldehyde phosphate} \rightleftharpoons \text{dihydroxyacetone phosphate}$$

The glyceric aldehyde phosphate is oxidized in a coupled reaction to 3-phosphoglyceric acid $\rightleftharpoons$ 2-phosphoglyceric acid. Initially α-glycerophosphate is formed by a reduction of a second molecule of triosephosphate. This type of reaction is called dismutation by Neuberg or more generally a Cannizzaro reaction. Subsequently the molecule reduced is not triose but a H_2-acceptor which forms later in the scheme (e.g., acetaldehyde, pyruvic acid). Enolase changes the phosphoglyceric acid into phosphopyruvic acid which is irreversibly dephosphorylated (Embden, Deuticke and Kraft, 1933) by the adenylic acid system, which may be regarded as a dissociable, active group of the enzyme, phosphorylase. The phosphate is carried by the adenylic acid (adenosinmonophosphate) as adenosindiphosphate or adenosintriphosphate, both discovered by Lohmann, to glucose which forms initially hexose-monophosphate and then the diphosphate (Harden and Young ester).

Needham and van Heyningen (1935) have reconstructed the

steps between phosphopyruvate and adenylic acid with the formation of adenylpyrophosphate and pyruvate and have shown that dephosphorylation of the phosphopyruvate is exothermic. Nature makes considerable use of phosphate transfer in connecting assimilation and dissimilation. Another reaction in which energy and phosphate are passed between molecules is that occurring in muscle extracts:

$$\text{Adenylpyrophosphate} + 2 \text{ creatine} \rightleftharpoons \text{Adenylic acid} + 2 \text{ creatinephosphate}$$

The heat of hydrolysis of adenylpyrophosphate is approximately equal to that required in the synthesis of creatinephosphate.

Creatinephosphate appears to serve as a reservoir for phosphate. It does not occur in yeast, however, and its rôle in bacterial metabolism is uncertain.

It is to be noted that the phosphate appears to work in a closed system although it has been demonstrated with muscle (Parnas and Ostern, 1936; Meyerhof, 1937), yeast (Macfarlane, 1936) and bacteria (Wiggert and Werkman, 1938) that inorganic phosphate is taken up and later released from organic combination. Needham and Pillai (1937) and Meyerhof (1937) have shown the change,

$$\text{Adenylic acid} + H_3PO_4 \rightarrow \text{adenylpyrophosphate}$$

to take place by the energy liberated from the following reaction:

$$\text{Pyruvic acid} + \text{triosephosphate} \rightarrow \text{lactic acid} + \text{phosphoglyceric acid} + 8{,}000 \text{ (approx.) cal.}$$

Pyruvic acid appears to be a general intermediary in bacterial dissimilation of glucose. It has been isolated in this laboratory in the case of a relatively large number of genera [*Propionibacterium* (Wood and Werkman, 1934), *Escherichia, Citrobacter, Aerobacter* (Reynolds, 1935), *Lactobacillus, Clostridium, Bacillus, Azotobacter,* among others (unpublished data)].

In the present state of our knowledge it appears that the Embden-Meyerhof-Parnas scheme of glycolysis finds application in the anaerobic dissimilation of bacteria. However, from experiments with NaF (Werkman, Stone and Wood, 1937; Wiggert

and Werkman, 1939) it is shown that the Embden-Meyerhof-Parnas scheme may not exclusively function in the dissimilation of glucose by *Propionibacterium pentosaceum* but that the organisms may possess in addition some other path of dissimilation. However, there are several facts which support the essential rôle of phosphoglyceric acid as a normal intermediary. First, it is readily isolated; secondly, it is dissimilated to normal final products (cf. Tikka, 1935). A third point is the behavior of other phosphate esters, i.e., phosphoglyceric acid can be isolated from the dissimilation of hexosediphosphate and hence the propionic acid bacteria must possess the enzyme systems requisite for glycolysis by the Embden-Meyerhof-Parnas scheme.

Further investigations may again reveal the greater versatility of bacteria as compared with the more differentiated cells of muscle and brain.

Dissimilation of pyruvic acid. Pyruvic acid may be looked upon as a cardinal intermediary in cellular metabolism. It has been shown to occur in the dissimilation of glucose by muscle, brain, kidney, yeast, fungi and bacteria. From it originate many products of cellular dissimilation such as, acetic, butyric, succinic and fumaric acids, ethyl alcohol, glycerol, acetylmethylcarbinol, 2,3-butylene glycol, acetone, isopropyl alcohol, carbon dioxide, and hydrogen. Pyruvic acid may also serve in the formation of amino acids. In muscle glycolysis the pyruvic acid is reduced to form lactic acid; with yeast it is first decarboxylated to acetaldehyde and CO_2, the former is then normally reduced to ethyl alcohol although when the fermentation is carried out at relatively alkaline pH levels, a dismutation of the acetaldehyde occurs to form ethyl alcohol and acetic acid in equimolar concentration (Neuberg's type III fermentation). Lipmann (1939) has shown that phosphate must participate in the dehydrogenation of pyruvic acid by lactic acid bacteria and the energy derived can be used to synthesize adenylic acid pyrophosphate from free phosphate and adenylic acid; Mg^{++}, Mn^{++} or Co^{++} is required. With bacteria the reactions involving pyruvic acid are more complex. Lactic acid (*d-*, *l-*, or *dl-*) may be formed by certain groups, i.e., *Lactobacillus*, *Streptococcus*, *Bacillus*; other groups

possess an essentially alcoholic mechanism, e.g., *Sarcina ventriculi* (Smit, 1930). A mixed lactic-alcoholic type of dissimilation is shown by *Thermobacterium mobile* which converts glucose into lactic acid (about 7 per cent), ethyl alcohol and CO_2 (45 per cent each) (Hoppenbrouwers, 1931). Other bacteria possess more complex mechanisms for the dissimilation of pyruvic acid.

Pyruvic acid may undergo the following changes anaerobically:

(1) $CH_3COCOOH \rightarrow CH_3CHO + CO_2$ (decarboxylation)

(2) $CH_3COCOOH + HOH \rightarrow CH_3COOH + HCOOH$ (hydrolysis)

(3) (1) followed by: $2\ CH_3CHO \begin{matrix} \nearrow\ CH_3COOH \\ \searrow\ C_2H_5OH \end{matrix}$ (dismutation)

(4) (1) followed by: $2\ CH_3CHO \rightarrow CH_3COCHOHCH_3$ (condensation and reduction)

$CH_3COCHOHCH_3 + 2\ H \rightarrow CH_3CHOHCHOHCH_3$

(5) $CH_3COCOOH + HOH \rightarrow CH_3COOH + 2\ H + CO_2$ (dismutation)

$CH_3COCOOH + 2\ H \rightarrow CH_3CHOHCOOH$

Reaction (1) is shown by both yeast and bacteria (*Sarcina ventriculi*) (Smit, 1930). Reaction (2) occurs apparently with many bacteria especially in the family *Bacteriaceae*. Organisms possessing hydrogenylase split the formic acid into H_2 and CO_2 (*Escherichia coli*) (Stephenson and Stickland, 1932), whereas in other species formic acid accumulates (*Eberthella* type). Reaction (3) is shown by yeast growing in a relatively alkaline medium (Neuberg and Hirsch, 1919), and probably by many bacteria. Reaction (4) may account for the formation of acetylmethylcarbinol and 2,3-butylene glycol by *Aerobacter* and yeast (Neuberg and Reinfürth, 1923). Reaction (5) is a dismutation demonstrated for the heterofermentative lactic acid bacteria by Nelson and Werkman (1936), confirmed by Krebs (1937a) for staphylococci and suggested by Quastel and Stephenson (1925) to account for the anaerobic dissimilation of pyruvic acid by *Escherichia coli*. The propionic acid bacteria bring about a dismutation of pyruvic acid to propionic acid (through lactic acid) and acetic acid and CO_2 (van Niel, 1928; Wood and Werkman, 1934).

Peters (1936) greatly increased our knowledge of the oxidation of pyruvic acid by his studies on the vitamin B_1-deficient pigeon

brain. The addition of thiamin stimulated the dissimilation of pyruvic acid. Lohmann and Schuster (1937) showed that the coenzyme of pyruvic acid decarboxylation was thiamin pyrophosphate; Lipmann (1937) then showed that thiamin pyrophosphate was involved in the dehydrogenation of pyruvic acid by an acetone preparation of *Bacterium acidificans-longissimum*. Hills (1938) reported a marked stimulation in the pyruvate metabolism of *Staphylococcus aureus* grown in thiamin-deficient media by simple addition of crystalline thiamin. Silverman and Werkman (1938, 1939a, 1939b) have shown that cell suspensions of *Propionibacterium pentosaceum* and *P. petersonii* require thiamin in the dissimilation of pyruvic acid and that there is an adaptation to the synthesis of thiamin by *P. pentosaceum* after growth in a thiamin-deficient medium. Barron and Lyman (1939) found gonococci, *Streptococcus hemolyticus* and *Staphylococcus aureus* to be stimulated in the breakdown of pyruvic acid by the addition of thiamin.

Although the dissimilation of pyruvic acid has been rather extensively studied, there is every reason to believe that there exist additional ways by which it functions in cellular metabolism of glucose. Wood and Werkman (1938) have suggested in connection with the utilization of carbon dioxide by heterotrophic bacteria that the formation of succinic acid involves pyruvic acid. They showed a direct correlation between the succinic acid formed and carbon dioxide fixed; furthermore, that sodium fluoride inhibits CO_2-fixation and that the formation of succinic acid is reduced to the same extent. This would suggest the possibility that the utilization of carbon dioxide involves a direct union with pyruvic acid. Perhaps this occurs even in photosynthesis. Should this suggestion be confirmed, it would offer an avenue for direct attack on problems of assimilation.

Aerobic dissimilation (respiration). Cellular respiration consists essentially of the dehydrogenation of the substrate molecule with transfer of hydrogen to gaseous oxygen through a series of oxidation-reduction systems involving primarily the transfer of electrons. The energy is thus liberated in a smooth continuous manner. The dehydrogenases adsorb the substrate molecules

and activate hydrogen which ultimately reaches molecular oxygen. Respiration is frequently defined so as to require the liberation of CO_2, i.e., a gaseous exchange. Such a definition cannot be rigidly adhered to since the liberation of CO_2 is purely incidental. Most respiring cells do evolve CO_2 although this is not always true. It is desirable to define respiration (aerobic dissimilation) in the sense of a process utilizing molecular oxygen as a hydrogen acceptor. The mechanism of bacterial respiration has received relatively little attention.

In the development of our concepts of respiration, the theory of the activation of the oxygen molecule was first generally accepted following the work of Bach. However, this theory of oxygen activation subsequently was found inadequate in itself. It has been revised by the Warburg school and conciliated with the Wieland theory of hydrogen activation almost simultaneously by Fleisch (1924), Szent-Györgyi (1924), Oppenheimer (1926) and Kluyver (Kluyver and Donker, 1926; Kluyver, 1931), independently. Kluyver particularly emphasizes the far-reaching effects and basic importance of the theory of the unity in the chemistry of cellular metabolism, and points out the general application of the theory of hydrogen transfer in biological oxidation both aerobic and anaerobic.

The investigations of Warburg have been concerned mainly with the rôle of iron in cellular respiration. According to his theory, iron in the form of a hematin derivative is intimately associated with the catalysis of respiration; the primary reaction is between iron and molecular oxygen and only in this manner can oxygen be used in respiration. Wieland regarded activation of the hydrogen as of primary importance, whereas Warburg (1928) believed the essential activation was that of the oxygen. Ionic iron was not effective, only iron in a complex organic form (Atmungsferment) was responsible for respiration. It is now known that the Atmungsferment is cytochrome oxidase of Keilin or very closely related. The inhibition of respiration by cyanide is plausibly explained by assuming a union of the iron compound and the cyanide to form an inactive complex. In fact Warburg developed his charcoal model on this premise. When active

charcoal is shaken with oxalic acid, an uptake of oxygen occurs with oxidation of the oxalic acid. The reaction is poisoned by cyanide or urethanes as in the case of living cells. The important point is that Warburg found the similarity between the charcoal model and the living cell lay in the presence of iron in both systems. When charcoals were made from pure cane sugar, the product was free from iron and inactive. Addition of iron salts, however, does not activate the charcoal; in addition organic nitrogen must be added along with the inorganic iron. The investigations of Warburg have done much to explain the utilization of oxygen by the living cell. They have been extended by the brilliant investigations of Keilin.

Systems of respiration. The simplest system of respiration will require a hydrogen donator (substrate), a dehydrogenase specific for the donator, and molecular oxygen as the H_2-acceptor. It is questionable whether organisms depending on such a system exist. Franke and Lorenz (1937) consider their "glucose-oxidase" which oxidizes glucose to gluconic acid to be an oxytropic dehydrogenase. Such a system is illustrated as follows, where AH_2 is the donator and O_2 the acceptor.

$$AH_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{dehydrogenase}} H_2O + A$$

This may be the case in the oxidation of amino acids, and aldehydes (Dixon, 1937). Some dehydrogenases not acting directly with O_2 do so through intervention of dyes (e.g., methylene blue) or naturally occurring pigments which require no activation to accept hydrogen and pass it on to oxygen. The dyes that have been used do not occur naturally, although many bacteria contain respiratory pigments, e.g., pyocyanine, phthiocol, chlororaphine and toxoflavin. This type of respiration may be illustrated by the following scheme, where C stands for the hydrogen-carrying dye or pigment.

$$(1)\ AH_2 + C \xrightarrow{\text{dehydrogenase}} CH_2 + A$$

$$(2)\ CH_2 + \tfrac{1}{2}\,O_2 \rightarrow C + H_2O$$

However, other systems cannot employ O_2 directly as an accep-

tor of hydrogen, although they may do so indirectly; these require specific carriers for the hydrogen. Such naturally occurring carriers themselves may require enzymic activation; e.g., coenzyme I (cozymase) requires activation by diaphorase (coenzyme-factor); flavoprotein requires no such activation. Certain of the carriers function in anaerobic dissimilation, whereas others function in connection with the transfer of hydrogen to molecular oxygen. It is the latter type of carrier in which we are now interested. Moreover, in the same cell, dehydrogenation of different donators may be brought about by different carriers or combinations of carriers. Perhaps the most widely distributed and important of the respiratory hydrogen carriers is cytochrome (Keilin, 1925).

Cytochrome-cytochrome oxidase system of Keilin. Largely disregarding the controversy between Wieland and Warburg, Keilin proceeded to conciliate and extend the views of the two investigators. Keilin's rediscovery of cytochrome and his investigations elucidating the properties and behavior of the cytochrome-oxidase system constitute the third in this series of classical investigations dealing with biological oxidation-reduction.

MacMunn in 1886, while investigating absorption spectra of tissues, described absorption bands of hematin compounds which are now known to have been those of cytochrome. Severe criticisms came, however, from Hoppe-Seyler (1890) and the initial discovery of cytochrome (called myo-hematin and histo-hematin by MacMunn) fell into disrepute and was not accepted until Keilin in 1925, rediscovered and named the respiratory haemochromogen, cytochrome.

Before discussing the rôle of cytochrome in bacterial respiration, it is desirable to make a few general remarks regarding cytochrome and its function in cellular respiration.

When a heavy suspension of aerobic or facultative bacteria under anaerobic conditions is examined spectroscopically, the four bands of cytochrome are plainly visible. Keilin regards cytochrome as consisting of three separate hemochromogens, cytochromes a, b, and c, each with two bands. Three of the bands (one from each of the three cytochromes) constitute nearly coin-

cident lines and comprise band d. Bands a, b and c are separate and belong to cytochromes a, b and c respectively. Cytochrome is a hemochromogen, i.e., it is a reduced hematin combined with a protein or other organic nitrogenous group. The important point is that cytochrome is oxidized and reduced in a living cell; it acts as a hydrogen carrier, passing the hydrogen on to molecular oxygen which has been activated by an enzyme formerly called indophenol oxidase and now known as cytochrome oxidase. Apparently, the union of hydrogen with oxygen is unusual in that no peroxide is formed. With respect to oxidation and reduction, the essential part of the cytochrome molecule is the iron which can undergo oxidation from the Fe^{++} to the Fe^{+++} state. It is not pertinent at this point whether the oxygen unites with the cytochrome, perhaps it is sufficient to say that water is formed in the reduction of the oxygen by hydrogen present. The transfer of an electron from Fe^{++} to H^{+} results in Fe^{+++} and H (represented by oxidized cytochrome and water). Succinic acid is the principal donator (cf. Szent-Györgyi, 1937) in animal tissue, although it has recently been shown that hexose-monophosphate may function. Our knowledge of bacterial respiration is still too fragmentary to speculate on the occurrence of events in bacteria although succinic acid is of widespread occurrence and there is evidence of stimulation of washed cells by the addition of the succinic-fumaric acid system. Cytochrome is of general occurrence among aerobic and facultative bacteria (Tamiya and Yamagutchi, 1933; Yaoi and Tamiya, 1928; Yamagutchi, 1937; Frei, Riedmuller and Almasy, 1934) and absent from most if not all strict anaerobes. Such facultative organisms as the propionic acid bacteria contain the cytochrome-oxidase system and it appears probable that continuous culture of such forms in the presence of oxygen leads to an increased activity of the cytochrome-oxidase system.

The function of cytochrome may be illustrated as follows:

(1) $AH_2 + \text{cytochrome c} \xrightarrow{\text{dehydrogenase}} A + \text{reduced cytochrome c}$

(2) $\text{Reduced cytochrome c} + \frac{1}{2}O_2 \xrightarrow{\text{oxidase}} \text{cytochrome c} + H_2O$

The reduced form of cytochrome c unlike other hemochromogens is not oxidized spontaneously by oxygen but requires the action of a specific enzyme, cytochrome oxidase. The absorption spectrum of reduced cytochrome may be clearly distinguished in active cultures of bacteria, even in such essentially anaerobic forms as the propionic acid bacteria.

In remaining types of respiration the hydrogen may be transferred through two or more carriers. Such systems may involve flavoprotein, i.e., "yellow enzyme" of Warburg. Warburg's (cf. Warburg and Christian, 1938) terminology has been criticised by Thunberg (1935) and Ogston and Green (1935a). For a discussion and comparative table of nomenclature see Dixon (1939).

Flavoprotein. Flavoprotein comprises a protein carrier with a prosthetic group discovered by Banga and Szent-Györgyi (1932) who called it cytoflav (cf. Laki, 1933) and suggested that it played a rôle in respiration. Our knowledge of the action of flavoprotein is due largely to the work of Warburg and Christian (1933) who isolated the conjugated protein from yeast and showed that the prosthetic group is lactoflavin-5-phosphoric acid (also called riboflavin phosphate or alloxazine nucleotide) and that it does not function efficiently as a carrier until combined with a protein. The active nucleus of the prosthetic flavinphosphate group is iso-alloxazine. Flavin is iso-alloxazine combined with ribose and is commonly called riboflavin.

Kuhn, Rudy and Weygand (1936) synthesized the prosthetic group; and by combining it with Theorell's protein (1935b) Kuhn and Rudy (1936a, 1936b) showed that the complex was active.

Reduced flavoprotein is spontaneously oxidized by molecular oxygen when no other electroactive system of higher potential is present (cf. Warburg and Christian, 1933). Normally it transmits its hydrogen to oxygen by way of cytochrome c when present, since the latter step is much more rapid at the lowered oxygen tensions presumably present in the cells (Theorell, 1936). The flavoprotein functions with specific dehydrogenases which require hydrogen carriers between the substrate and the flavoprotein.

According to Ogston and Green (1935a), flavoprotein reacts with greatest activity, as a hydrogen carrier, with hexose diphosphate, hexosemonophosphate, glucose and malate as donators. The normal behavior of flavoprotein may be represented:

$$AH_2 + \text{coenzyme} + \text{flavoprotein} \xrightarrow{\text{dehydrogenases}} H_2\text{-flavoprotein} + A$$

$$H_2\text{-flavoprotein} + \text{cytochrome c} \rightarrow \text{flavoprotein} + H_2\text{-cytochrome c}$$

$$H_2\text{-cytochrome c} + \tfrac{1}{2}\,O_2 \xrightarrow{\text{oxidase}} H_2O + \text{cytochrome c}$$

In the presence of cyanide inhibition of the cytochrome oxidase, flavoprotein may perhaps, transmit hydrogen directly to oxygen although Theorell considers such a transfer as unphysiological. Since *L. delbrueckii* contains no cytochrome which can serve to accept hydrogen from the flavoprotein, its reoxidation probably depends on anaerobic hydrogen acceptors in the case of these bacteria. Flavoprotein appears to act as a hydrogen carrier between slowly reacting systems and its rôle in anaerobic dissimilation is probably that of a carrier. Coenzymes I and II reduce it. Since riboflavin phosphate can be reversibly oxidized and reduced, it behaves as an indicator.

Although previously only one flavoprotein was known, recently new ones have been discovered in which the prosthetic group is flavin-adenine-dinucleotide (coenzyme of amino acid oxidase). Haas (1938) has isolated a second flavoprotein from yeast in which the prosthetic group, like that in milk flavoprotein, is flavin-adenine-dinucleotide and the protein carrier differs from that of the original yeast flavoprotein and with which lactoflavin-phosphate is inactive. Haas obtained both fractions separately and was able to combine them to form the active flavoprotein.

Diaphorase, the dehydrogenase of coenzyme I, recently isolated by Straub (1939) and Straub, Corran and Green (1939) is a flavoprotein. The presence in animal tissue of an enzyme catalyzing the oxidation of reduced coenzymes I and II was demonstrated by Adler, Das and Euler (1937) and Dewan and Green (1938). The latter have demonstrated the presence of diaphorase in *E. coli*, *Bacillus subtilis* and *Bacterium proteus*.

Flavoprotein has been spectroscopically estimated in a number of genera, e.g., *Lactobacillus*, *Acetobacter*, *Clostridium*, *Flavobacterium*, *Escherichia* (Schütz and Theorell, 1938). Its occurrence in cells is general. Schütz and Theorell (1938) observed that *Lactobacillus delbrueckii*, *E. coli*, *Streptococcus lactis* and *Flavobacterium lacticum* did not alter their flavoprotein content when subjected to aerobic and anaerobic conditions, a finding which supports the view of Theorell (1936) that physiologically flavoprotein does not react directly with oxygen.

Wood, Andersen and Werkman (1938) have shown riboflavin to stimulate growth of *Lactobacillus* and *Propionibacterium* when added to a medium deficient in this constituent. Doudoroff (1938–39) has shown a similar stimulation of luminescent bacteria.

The investigations of Szent-Györgyi (1937) and of Krebs (1937b) have led to recognition of still another type of respiration based on fumaric acid catalysis. In the work of Szent-Györgyi, it was found that succinic acid was rapidly dehydrogenated to fumaric acid through cytochrome. These investigators believe that fumaric acid is a H_2-carrier along with oxalacetic acid. According to Krebs, fumaric acid is a carrier in the respiration of certain bacteria such as *Escherichia coli* and *Staphylococcus aureus*.

If fumaric acid is a carrier, it should, if present in sufficient quantity, carry on the oxidation of the substrate in the absence of oxygen and be recovered as succinic acid; and the rate of reaction should not be less than that in the presence of free oxygen. Furthermore, it must be shown that succinic acid occurs under physiological conditions in the dissimilation of the substrate and is oxidizable in the presence of oxygen.

The rate of the reaction can usually be determined by measuring CO_2 evolved or substrate disappearing. The reactions may be set up as follows:

(1) Glucose + fumarate $\rightarrow$ CO_2 + succinate
(2) Succinate + $\frac{1}{2}O_2$ $\rightarrow$ fumarate + H_2O

Reaction 1 must occur as rapidly as number 2.

Table 1 taken from Krebs (1937b) illustrates the point.

The final step is to show that the succinate formed will transport hydrogen to O_2 as rapidly as glucose since no reaction can

limit the rate to less than that of glucose. In fact, the rate of transport of hydrogen to oxygen from succinate is greater than the rate of glucose breakdown. Since this is true, some other reaction must limit the breakdown of glucose.

An objection which has been raised against the theory of Szent-Györgyi is based on the well known fact that the 4-carbon dicarboxylic acids are intermediary products in carbohydrate metabolism and are burned in the cell, the inference being that irre-

TABLE 1

Rates of oxidation of glucose by Bacterium coli by molecular O_2 and by fumarate (Krebs, 1937b)

40°; pH 6.8; 2 mg. (dry weight) bacteria per flask.

TIME AFTER THE ADDITION OF 0.507 MGM. GLUCOSE	CO_2 PRODUCED (μl.)	
	In the presence of O_2	Anaerobically in the presence of M/20 fumarate
minutes		
10	60.5	78
20	139	162
40	219	198
60	237	223
80	246	245
100	254	262
120	263	280
Succinic acid* found in the solution (μl.).		510

* One millimol = 22,400 μl.

versibly oxidized substances cannot function as catalysts. However, we have found a catalytic effect in the metabolism of *Micrococcus lysodeikticus* at low concentrations of fumaric acid. The increase in oxygen taken up exceeded by several times the quantity required to oxidize the fumaric acid added.

The succinate is reoxidized to fumarate by the cytochrome-oxidase system. The type may be represented:

$$AH_2 \xrightarrow{\text{dehydrogenase}} \text{coenzyme I} \xrightarrow{\text{diaphorase}}$$

$$\text{fumarate} \xrightarrow[\text{dehydrogenase}]{\text{succinic}} \text{cytochrome} \xrightarrow{\text{oxidase}} O_2$$

$$\text{flavoprotein} \longrightarrow O_2$$

Although cell respiration is the sum total of oxygen used by all types present in a cell, there is practically no knowledge of the proportional respiration accounted for by the different mechanisms.

PRINCIPAL AND SECONDARY RESPIRATION

From the standpoint of the cell, respiration is frequently classified either as (1) principal and secondary or (2) cyanide-sensitive and cyanide-insensitive. Principal respiration (Hauptatmung) is that constituting the main types in a normal cell, whereas secondary respiration (Nebenatmung) is that which constitutes relatively incidental (non-ferment hemin) respiration. In the main, principal respiration is that due to ferment-hemin (cytochrome) and is cyanide-sensitive although part of the secondary is also sensitive. The principal respiration probably includes certain reactions such as that due to flavoprotein which normally brings about oxidation through the cytochrome-oxidase system but which may transmit hydrogen directly to oxygen when the cytochrome is poisoned by cyanide. Alt (1930) has shown that cyanide-insensitive respiration is less in normal cells than in those that have been injured during the experiment. It is frequently claimed that this observation proves the abnormal character of such secondary respiration. The finding of Gourévitch (1937) that the amount of extractable flavin is proportional to cyanide-insensitive respiration indicates that this secondary respiration is due to a change in the behavior of the "yellow respiratory enzyme" to react directly with oxygen after inhibition by cyanide. It is probable that a substantial part of the secondary respiration of bacteria consists of pigment respiration. Present in bacteria are many pigments which function as carriers or acceptors, accepting hydrogen and passing it on perhaps directly or indirectly to molecular oxygen. Among the better known are pyocyanine, phthiocol and toxoflavin not to mention those such as the flavins which have been more thoroughly discussed already. Pyocyanine according to Friedheim (1931) strongly stimulates the respiration of pigment-free strains of *Pseudomonas aeruginosa* as well as of red blood cells. He found a more marked effect on

cells containing hemin systems. Such catalysis is cyanide-sensitive. Reduced pyocyanine is autoxidizable and in those cases where the oxidation is brought about by direct reaction with molecular oxygen (without iron) cyanide will exert no effect; e.g., pyocyanine respiration of the anaerobic tetanus bacteria (Frei, 1934). When pyocyanine acts as the H_2-acceptor in the respiration of bottom yeast with hexosemonophosphate as the substrate, cyanide (M/600) causes an inhibition of 31 per cent of the pyocyanine catalysis (Ogston and Green, 1935a, 1935b). The respiration of *Staphylococcus aureus* is strongly stimulated by pyocyanine (Ehrismann, 1934).

Phthiocol is a yellow pigment found in the tubercle bacillus. It exists in the bacteria in oxidized form and is a relatively negative redox system. Its effect on respiration has not been reported. *Bacterium violaceus* contains a pigment, violacein, which increases respiration of bacteria freed of their pigment. Since the reduced form is not autoxidizable, it must function as a hydrogen carrier according to Friedheim (1932). Chlororaphin, the green pigment of *Bact. chlororaphis* forms a reversible system sharing many characteristics with pyocyanine (Elema, 1933). Toxoflavin found in *Bact. bongkrek* (van Veen and Mertens, 1934) is the prosthetic group of a toxic yellow pigment and is a reversible system, electroactive between pH 4 to 8 (Stern, 1935). Phoenicein found in *Penicillium phoeniceum*, is a reversible redox system. The reduced form is autoxidizable. The fungus contains no cytochrome and its respiration may be iron-free. Phoenicein increases the respiration of *Ps. aeruginosa* several times (Friedheim, 1933). In addition, secondary oxidations by H_2O_2 with peroxidases and simple iron systems involving direct attack on the substrate may occur. These would be part of the secondary respiration but cyanide-sensitive, since peroxidases have been shown to contain iron. Finally, as part of secondary respiration there may occur a direct oxidation of unsaturated linkages especially in the fatty acids by hemin (Kuhn and Brann, 1927) or metal salts (Rosenthal and Voegtlin, 1933).

Haas (1934) has given us evidence in the case of yeast, regarding the relative intensities of the principal and secondary respirations.

Using a spectroscopic method, Haas demonstrated that in the intact cells practically all of the oxygen consumption is due to the activity of the cytochrome system.

Roman (1938) lists under "Nebenatmung"

A. Dehydrogenations
   (1) Oxytropic dehydrogenation (Schardinger reaction).
   (2) Flavoprotein
   (3) Oxyhydrogenases and oxidases: oxidative deamination of amino acids, protamines, tyrosine and uric acid.
   (4) Quinone catalyses by autoxidizable chromogens.
   (5) Secondary oxidations by H_2O_2.
      (a) Direct on the substrate.
      (b) By peroxidases and thermostable iron systems (pseudo-peroxidases).

B. Non-dehydrogenation (peroxide) oxidation as with unsaturated fatty acids; perhaps further little-known mechanisms, i.e., ω-oxidation and ring splitting (proline).

Studies on cellular metabolism have confirmed the view, originally proposed by Wieland and conciliated with the view of Warburg, especially by Kluyver, that the essential principle of cellular metabolism involves the transference of hydrogen from donator to acceptor. A series of graduated redox systems provides the mechanism of transfer and results in a smooth and regulated flow of energy for the use of the cell in assimilation. Under anaerobiosis, the ultimate transfer is limited to reducible intermediate products of dissimilation; under aerobiosis, oxygen is the final acceptor. Here the systems of Warburg and Keilin are active. Hydrogen, activated by suitable dehydrogenases and transported by carrier systems, is accepted by the inert oxygen only after activation of the oxygen molecule. This is accomplished by alternative reduction of the Fe^{+++} in the prosthetic group of hemin compounds by the hydrogen, and oxidation of the Fe^{++} by the oxygen from the air. This path must constitute, in the main, that of the principal respiration; in addition a certain uptake of oxygen occurs in the absence of the hemin compounds. Our knowledge of bacterial respiration is too inadequate to reconstruct the respiratory systems with any degree of assurance. In

the light of our present knowledge, it appears that the principles and the basic mechanisms underlying bacterial dissimilation are substantially those found in the higher, differentiated forms of life but providing for greater adaptation and variation.

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